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# Enzyme–microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*

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**Specific cellulose hydrolysis rates (g of cellulose/g of cellulase per h) were shown to be substantially higher (2.7- to 4.7-fold) for growing cultures of *Clostridium thermocellum* as compared with purified cellulase preparations from this organism in controlled experiments involving both batch and continuous cultures. This “enzyme–microbe synergy” requires the presence of metabolically active cellulolytic microbes, is not explained by removal of hydrolysis products from the bulk fermentation broth, and appears due to surface phenomena involving adherent cellulolytic microorganisms. Results support the desirability of biotechnological processes featuring microbial conversion of cellulosic biomass to ethanol (or other products) in the absence of added saccharolytic enzymes.**

cellulase | cellulosome | consolidated bioprocessing | ethanol

**P**roduction of fuels from cellulosic biomass could offer large benefits in terms of sustainability, security, and rural economic development [refs. 1 and 2; Greene *et al.* (2004), [www.nrdc.org/air/energy/biofuels/biofuels.pdf](http://www.nrdc.org/air/energy/biofuels/biofuels.pdf)]. The cost of producing reactive intermediates from cellulosic biomass is the central technical obstacle to be overcome for these benefits to be realized (3). Accordingly, a substantial and ongoing effort has been devoted to describing the components and mode of action of multicomponent cellulase enzyme systems (4–8), and to improving their effectiveness (9–11). Synergism among cellulase components is an important and widely documented phenomenon in this context. Values for the degree of synergism (DS, the activity exhibited by mixtures of components divided by the sum of the activities exhibited by the components acting separately) >2 are common, and DS values >5 have been reported under some conditions (8).

Cellulose hydrolysis can be mediated by cellulase enzymes acting in the absence of cells, by cellulases acting in the presence of cells but with no cell–enzyme attachment, or by cellulases attached to cells. In the latter case, hydrolysis is mediated by ternary cellulose–enzyme–microbe (CEM) complexes rather than binary cellulose–enzyme (CE) complexes. For anaerobic cellulolytic bacteria, CEM complexes are commonly formed and are thought to be the major agent of cellulose hydrolysis (12). Potential benefits of CEM complexes for cellulolytic microorganisms have been suggested, including preferred access to hydrolysis products and local concentration of cellulases (12–16).

The possibility of enhanced effectiveness of cellulases present in CEM complexes has been mentioned (12) but has not been demonstrated or quantitatively evaluated. We investigate such “cell–enzyme synergy” here for *Clostridium thermocellum*, an anaerobic thermophilic bacterium that exhibits one of the highest rates of cellulose utilization among described microorganisms (12). *C. thermocellum* produces a cellulase complex, or “cellulosome,” a substantial fraction of which is bound to the cell surface under most culture conditions (17–20). Recently, Zhang and Lynd (21) showed that *C. thermocellum* assimilated cello-dextrins with a mean degree of polymerization of  $\approx 4$  during growth on cellulose, and that these cello-dextrins were subsequently cleaved by intracellular phosphorylolytic enzymes. This organism is a starting point for developing anaerobic microbes capable of one-step processing of cellulosic biomass to ethanol

or desired products in the absence of added saccharolytic enzymes (3, 12, 22).

## Results

Hydrolysis of microcrystalline cellulose (Avicel, FMC, Philadelphia, PA) was compared in batch and continuous cultures for two systems.

- Growing cultures of *C. thermocellum* in the absence of added cellulase, “microbial hydrolysis,” in which hydrolysis is mediated by both CEM and CE complexes.
- Enzymatic hydrolysis mediated by purified *C. thermocellum* cellulosome with fermentation of hydrolysis products by the noncellulolytic thermophilic anaerobe *Thermoanaerobacterium thermosaccharolyticum*. In such simultaneous saccharification and fermentation (SSF), hydrolysis is mediated by CE complexes only.

In batch culture under the conditions examined, microbial hydrolysis required 16 h for complete hydrolysis of 2 g of cellulose/liter, during which the cellulosome and cell protein concentrations roughly doubled from 48 to 98 and 125 to 264 mg/liter, respectively (Fig. 1A). SSF required 32 h for complete hydrolysis, during which the cellulosome concentration was 100 mg/liter, and the cell protein concentration increased from 160 to 260 mg/liter (Fig. 1B). The conditions under which microbial hydrolysis and SSF occurred were very similar (see Figs. 4 and 5, which are published as supporting information on the PNAS web site). The same growth medium and pH were used, and the concentrations of fermentation products were low. Hydrolysis products (total soluble glucans) in the growth medium were  $\leq 0.02$  g/liter at all times for both microbial hydrolysis and SSF, 2 orders of magnitude less than concentrations at which 50% inhibition of the *C. thermocellum* cellulase system is observed (23). The cellulase-specific activity was quite similar for enzymatic hydrolysis and microbial hydrolysis (measured with metabolically inactive cells) and remained nearly constant throughout the experiment (Table 2, which is published as supporting information on the PNAS web site).

Cellulose concentration is plotted vs. time in Fig. 2 for microbial hydrolysis (from Fig. 1A), SSF (from Fig. 1B) and for controls as follows: cell-free control 1, 100 mg/liter purified cellulase with no fermenting organism; microbial control 1, a *C. thermocellum* culture (100 mg/liter cellulosome, 264 mg/liter cell protein) with 38.5 mM sodium azide, a fermentation inhibitor; cell-free control 2, as for cell-free control 1 with 38.5 mM

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Abbreviations: CEM, cellulose–enzyme–microbe; CE, cellulase–enzyme; SSF, simultaneous saccharification and fermentation; CBP, consolidated bioprocessing.

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setting is a challenging proposition for biotechnology. However, there are increasing indications that such development is both desirable and achievable. Results presented herein suggest that cellulose hydrolysis may be more rapid when mediated by adherent cellulolytic microbes in a consolidated bioprocessing (CBP) configuration as compared with cellulase enzymes acting independently of cellulolytic microbes. The feasibility of CBP is supported by our recent finding that *C. thermocellum* realizes bioenergetic benefits specific to growth on cellulose that exceed the bioenergetic cost of cellulase synthesis (21). Process design studies indicate that CBP represents a potential breakthrough for low-cost processing of cellulosic biomass (3).

If development of a CBP-enabling microorganism is to be based on naturally occurring cellulolytic microorganisms such as *C. thermocellum*, it will be necessary to perform metabolic engineering to improve product yield and titer relative to performance obtained to date with available strains. Substantial advances have recently been made in developing requisite genetic tools for this approach (29, 30). Alternatively, organism development could be based on a microbe that already produces high product yields and titers (e.g., yeast) but is not cellulolytic. In this case, it will likely be desirable to understand, incorporate, and perhaps enhance physiological features that underlie enzyme-microbe synergy. Progress and prospects for both CBP organism development strategies are reviewed in detail elsewhere (3, 12).

## Materials and Methods

**Microbial Cultures and Chemicals.** *C. thermocellum* ATCC 27405 was provided by Arnold Demain (Massachusetts Institute of Technology, Cambridge, MA) and has been maintained in our laboratory, as described (31). *T. thermosaccharolyticum* ATCC 31960 was purchased from American Type Culture Collection (Manassas, VA). Chemically defined MTC medium was prepared as described (18) with changes as noted below. All chemicals were reagent grade and were obtained from Sigma (St. Louis, MO), unless indicated otherwise.

**Batch Culture of *C. thermocellum* and Relevant Controls.** Five milliliters of a *C. thermocellum* stock culture was inoculated by syringe into 100 ml of MTC medium containing 2 g/liter Avicel PH105 and 10 g/liter 4-morpholinepropanesulfonic acid buffer (initial pH 7.6) in triplicate 200-ml sealed serum vials (Bellco Biotechnology, Vineland, NJ) under an N<sub>2</sub> atmosphere. Cultures were incubated at 60°C in a water bath with rotary shaking at 200 rpm. Once 2 g/liter Avicel was consumed, as determined by visual inspection, supplemental Avicel was added by syringe as a 40 g/liter sterile suspension to a concentration of 2 g/liter, the pH was adjusted to 7.6 by addition of 4 M NaOH, and the gas phase was replaced by flushing with filter-sterilized N<sub>2</sub>. The microbial cellulose utilization data presented in Figs. 1 and 2 and Table 1, as well as Fig. 8, which is published as supporting information on the PNAS web site, are taken with the initial (time 0) data point just after supplemental Avicel addition. Microbial control 1 was carried out as above except that a sterilized 1 M sodium azide solution was added to a final concentration of 38.5 mM in conjunction with supplemental Avicel addition. Addition of azide as specified above resulted in cessation of fermentation as indicated by constant concentrations of fermentation products over time measured by HPLC.

**Cellulase Preparation and Purification.** Cellulase used for batch SSF experiments was obtained from batch cultures of *C. thermocellum* grown in MTC medium in a 200-ml serum vial with Avicel as the growth substrate at an initial concentration of 4 g/liter. Cellulase for continuous SSF experiments was obtained from steady-state continuous cultures of *C. thermocellum* grown in

MTC medium at a dilution rate (flow rate/fermentor working volume) of 0.052 h<sup>-1</sup> and feed cellulose concentration of 4 g/liter. Purification of cellulase from culture supernatants was carried out by affinity digestion as described (18, 32). Purified cellulase preparations used for batch and continuous SSF experiments contained ≈1.2 g/liter cellulase with a specific activity of 2.8 units/mg of cellulase in Tris buffer (50 mM, with 10 mM CaCl<sub>2</sub>, pH 6.8). The concentration of soluble hydrolysis products in the purified cellulase preparation was verified by HPLC to be sufficiently small (<0.002 g/liter) to not complicate the interpretation of SSF experiments. The cellulase enzymes used for SSF experiments may not be exactly the same as those present in growing *C. thermocellum* cultures. However, several factors suggest that any differences between the cellulases present under the conditions examined are not large and do not confound the interpretation of synergy as presented herein (see *Supporting Text*, which is published as supporting information on the PNAS web site).

**Batch SSF and Relevant Enzyme Controls.** Five milliliters of a *T. thermosaccharolyticum* stock culture was inoculated into 100 ml of MTC medium containing 2 g/liter Avicel PH105 and 2 g/liter cellobiose in triplicate 200-ml serum vials under an N<sub>2</sub> atmosphere. Cultures were incubated at 60°C in a water bath with rotary shaking at 200 rpm. Once cellobiose was exhausted, as determined by HPLC, the pH was adjusted back to 7.6, and a purified cellulosome preparation (above) was sterilized by using a filter (Millex-GV, 0.22 μm pore size, Millipore, Billerica, MA) and added to the culture by syringe to a final concentration of 100 mg/liter. The SSF data presented in Figs. 1 and 2 and Table 1, as well as Fig. 5, are taken with the initial (time 0) data point just after cellulase addition. Cell-free control 1 was carried out in the presence of 2 g/liter Avicel and 100 mg/liter purified cellulase as above, except that no fermenting organism was present. Cell-free control 2 was carried out as for cell-free control 1, except that a sterilized 1 M sodium azide solution was added to a final concentration of 38.5 mM.

**Continuous Culture.** A modified 1-liter fermentor (Applikon Dependable Instruments, Foster City, CA, modified by NDS) with an overflow sidearm (i.d. 0.38 in.) and 0.5-liter working volume was used both for microbial fermentation by *C. thermocellum* and for SSF carried out in continuous mode. pH was controlled at 6.8 by a Delta V process control system (New England Controls, Mansfield, MA) with addition of 4 M NaOH, the fermentor was stirred at 250 rpm, and temperature was controlled at 60°C by circulating hot water through the fermentor jacket (19). MTC medium containing 4 g/liter Avicel PH was fed by a peristaltic pump as described elsewhere (31) to achieve residence times as specified in the text. For SSF experiments, an additional peristaltic pump was used to deliver purified cellulase in 50 mM Tris buffer (pH 6.8) and stored at 4°C. A diagram of the SSF system is presented in Fig. 8. The composition of MTC medium and the concentration of Avicel used for SSF experiments were adjusted to provide the same concentrations as those used in *C. thermocellum* fermentation experiments (e.g., final concentration of 4 g/liter Avicel). SSF experiments were initiated by inoculating 50 ml of a late-exponential phase culture of *T. thermosaccharolyticum* into MTC medium containing 4 g/liter Avicel supplemented with 2 g/liter cellobiose. Once growth was evident, cellulase addition was commenced. Samples used to calculate steady-state values for continuous fermentations were taken at intervals of at least one residence time. Results are reported as the average of at least four steady-state samples.

